FEBS 15002
FEBS Letters 358 (1995) 31–33

The 'CheA' and 'CheY' domains of *Myxococcus xanthus* FrzE function independently in vitro as an autokinase and a phosphate acceptor, respectively

Gonzalo Acuña**, Wenyuan Shi, Kathy Trudeau, David R. Zusman*

Department of Molecular and Cell Biology, 401 Barker Hall, University of California, Berkeley, CA 94720, USA

Received 17 October 1994; revised version received 5 December 1994

Abstract **FrzE is a chemotaxis protein in *Myxococcus xanthus* which has sequence homology to two different chemotaxis proteins of enteric bacteria, CheA (autokinase) and CheY (phosphate acceptor) [Proc. Natl. Acad. Sci. USA 87 (1990) 5898–5902]. It was also shown that a recombinant FrzE protein was autophosphorylated when incubated in the presence of ATP and Mn^{2+} [J. Bacteriol. 172 (1990) 6661–6668]. In this study, we further investigated the biochemical properties of FrzE. Two recombinant proteins were produced: one containing only the 'CheA' domain of FrzE and the second only the 'CheY' domain. The CheA domain polypeptide contained the autokinase activity which was absent from the CheY domain polypeptide. The phosphorylated CheA domain polypeptide as well as the intact FrzE protein were able to transfer phosphate groups to the CheY domain peptide. These results indicate that FrzE has structural as well as functional homologies to CheA and CheY in a single polypeptide.**

Key words: Chemotaxis; Signal transduction; Two-component system; *frz*; *Myxococcus xanthus*

1. Introduction

Myxococcus xanthus is a Gram-negative gliding bacterium that exhibits chemotactic behavior which is necessary for colony swarming and aggregation during fruiting body formation [[1,2). Chemotaxis in *M. xanthus* requires a group of genes called *frz*, which have structural homology to the chemotaxis (*che*) genes of enteric bacteria [1,2]. For example, FrzA shows homology to CheW, FrzCD is homologous to Tar (a methyl-accepting chemotaxis protein, MCP), FrzE is homologous, in separate domains, to CheA and CheY, FrzG is homologous to CheB, and FrzF is homologous to CheR; FrzB shows no apparent homology to other previously identified proteins.

In enteric bacteria, CheA and CheY are known to play a central role in the signal transduction pathway of chemotaxis. These proteins belong to the family of two component regulatory proteins [3,4]: CheA is a histidine kinase that autophosphorylates in the presence of ATP and divalent cations and then transfers the phosphate group to an aspartate residue of CheY. Phospho-CheY then interacts with the switch components of the flagellar motor resulting in a change of rotation of the flagella [5]. FrzE of *M. xanthus* is homologous to both CheA and CheY [6]. The amino acid sequence of the N-terminal region of FrzE shows a conserved stretch of 20 amino acids which is homologous to the putative histidine phosphorylation site of CheA and a large central region of 360 amino acids which shows 66% amino acid homology to CheA; between the two regions there is an unusual alanine and proline rich region which may serve as a flexible hinge [6]. The C-terminal region of FrzE contains a 124-amino-acid domain which shows 60% amino acid homology to CheY and contains a region homologous to the aspartate phosphorylation site [6]. Polyclonal antibodies raised against the complete FrzE protein when used to analyze Western blots of *M. xanthus* extracts, showed that FrzE is present only as a single 83 kDa protein [7], and not as two smaller peptides. Thus, the two domains of FrzE are part of a single protein and are not translated as two separate polypeptides or processed posttranslationally into separate peptides. In this paper, we prepared recombinant polypeptides containing only one of the domains and studied the biochemical nature of each of these separated domains. Our results indicate that FrzE has dual functions: the CheA domain of FrzE is an autokinase and the CheY domain is a phosphate acceptor.

2. Materials and methods

Escherichia coli strain DH5αF′ (F′/*endA1 hsdr17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF) U1679 deoR (φ80dlac Δ(lacZ) M15)*) [8] was used for plasmid growth and maintenance. *E. coli* strain CJ236 (F′*cat dut ung1 thi-1 relA1 spoT1 mcrA*) [8] was used for site directed mutagenesis. *E. coli* strain BL21 (DE3) (F⁻ *ompT hsdSB*, with a prophage carrying the T7 RNA polymerase gene) [8] was used for gene expression with the T7 promoter vectors. This strain contains a single chromosomal copy of the phage T7 RNA polymerase gene under control of the *lacUV5* promoter.

Fig. 1 shows the strategy used for the construction of plasmids for overexpressing the recombinant proteins. Plasmid pGA31 (for overexpressing FrzE) was made from plasmid pBM33 [7] by digesting the plasmid with *Not*I and *Pst*I, removing the overhanging ends with T4 DNA polymerase, and religating it (Fig. 1a). A 464 bp *Sau96*I fragment containing the CheY domain of FrzE was isolated from the plasmid pBM33 [7] and cloned, in frame, in the *Stu*I site of the vector pMalc (New England Biolabs) (Fig. 1b). The resulting plasmid, pGA22, expressed a fusion gene consisting of the *E. coli* maltose binding protein (MBP) and the CheY domain of FrzE (called MBP:FrzE-CheY in this paper). The MBP:FrzE-CheY has a molecular mass of 54.5 kDa; and it carries a specific and unique recognition site for the factor Xa protease at the fusion junction. The expression vector for the CheA domain of FrzE (pGA34) was constructed by site directed mutagenesis. For this purpose, the *Sph*I/*Sal*I fragment of *frzE* was subcloned in the vector pBLKS+ (Fig. 1c) which allowed the synthesis of single stranded DNA necessary for the site directed mutagenesis procedure. The resulting plasmid, pGA25, was subjected to site directed mutagenesis by which the *frzE* amino acid codon CCC (Proline at 654) was changed to the stop codon UGA. The resulting mutants were screened by DNA sequencing. The mutated region, contained within a *Bgl*II/*Sal*I fragment,

*Corresponding author. Fax: (1) (510) 642 7000.

**Present address:* Givaudan-Roure Research Ltd., CH-8600 Dubendorf, Switzerland.

SSDI 0014-5793(94)01389-6

was used to replace the corresponding wild type fragment in pGA31 (Fig. 1d).

Isolation and purification of FrzE and FrzE-CheA were performed as described [7] with some minor modifications. Briefly, the plasmids pGA31 and pGA34 were transformed into the strain BL21(DE3). The cells were grown to early exponential phase and induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG). After 2 h of induction, the cells were harvested by centrifugation, washed once with 10 mM HEPES buffer, pH 7.2, containing 5 mM EDTA, and disrupted by sonication. The extracts were then centrifuged at 8,000 × *g* for 10 min, and the pellets washed three times with buffer containing 10 mM HEPES pH 7.2, 5 mM EDTA, and 0.1% Triton X-100. The pellets were solubilized and denatured in 6 M guanidine hydrochloride, 50 mM HEPES, pH 7.2. The denatured proteins were slowly renatured by dialysis against 10 mM HEPES pH 7.2, 1 mM DTT, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 50% Glycerol.

The MBP: FrzE-CheY polypeptide was overproduced in *E. coli* using the vector pMalc. In this vector, transcription is initiated from the *tac* promoter and is induced by IPTG. The protein was purified from crude lysates using affinity chromatography according to the protocol provided by New England Biolabs. However, attempts to specifically cleave the fusion protein with factor Xa protease gave very low yields. Therefore, most of the phosphorylation assays were carried out with the fusion protein since it retained the ability to be phosphorylated.

3. Results and discussion

Purified FrzE, FrzE-CheA, and MBP: FrzE-CheY (0.2 mg of each protein) were each added to a reaction buffer consisting of 50 mM HEPES, pH 7.2, 10 mM $MnCl_2$, 10 mM $MgCl_2$, and 0.1 mM DTT. The phosphorylation reactions were carried out by the addition of 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) to the reaction buffer followed by incubation at 22°C for 10 min. The reactions were stopped by the addition of 3 ml of 4× stop buffer (0.32 M Tris-HCl, pH 6.8, 0.1 M EDTA, 8% SDS, 40% glycerol). The phosphorylated proteins were analyzed as follows: The reaction mixtures were subjected to 10% SDS-polyacrylamide gel electrophoresis. The proteins were then blotted onto nitrocellulose membranes with a semidry blotting apparatus and the membranes subjected to autoradiography. Fig. 2 (lane 1) shows that FrzE was autophosphorylated when incubated in the presence of ATP and Mn^{2+}, as reported previously [7]. FrzE-CheA was also autophosphorylated by ATP (Fig. 2, lane 2) and was Mn^{2+} dependent (data not shown). Fig. 2 also shows that MBP: FrzE-CheY did not have any autokinase ac-

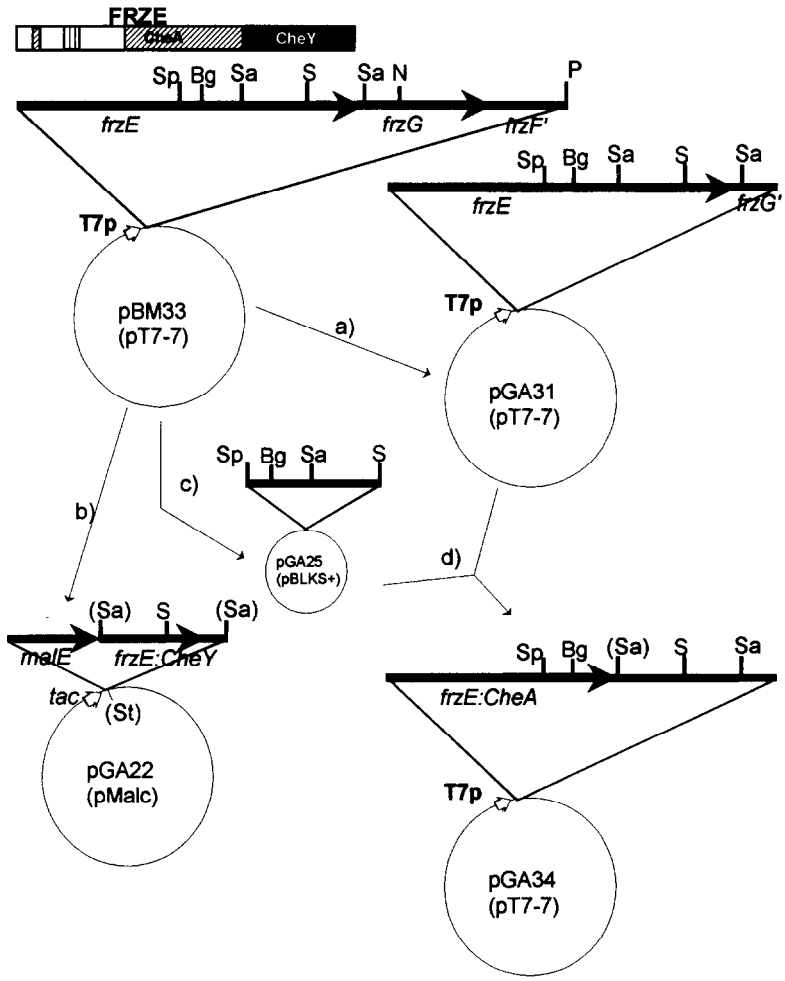

Fig. 1. Construction of the expression plasmids. The scheme on top represents the FrzE protein and the homology regions to the corresponding Che proteins. The CheA homologous region is shown as a stripped box; the putative hinge region is shown as a box filled with vertical lines; and the CheY homologous region is shown as a filled box. Only the relevant restriction sites are indicated. Restriction sites shown in parenthesis represent sites lost during the cloning steps. Each cloning step a, b, c, and d is explained in detail in the text. Abbreviations: Bg = *Bgl*II, N = *Not*I, P = *Pst*I, S = *Sal*I, Sa = *Sau96*I, Sp = *Sph*I, St = *Stu*I, T7p = T7 RNA polymerase promoter, *tac* = *tac* promoter.

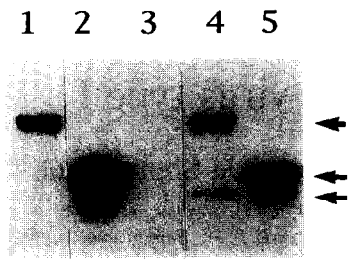

Fig. 2. Phosphorylation activities of FrzE, FrzE-CheA and MBP:FrzE-CheY in the presence of ATP and Mn^{2+}. See text for detailed experimental procedures. Lane 1, FrzE; lane 2, FrzE-CheA; lane 3, MBP:FrzE-CheY; lane 4, FrzE + MBP:FrzE-CheY; lane 5, FrzE-CheA + MBP:FrzE-CheY. The arrows represent the following proteins respectively (top tp bottom): FrzE, FrzE-CheA, and MBP:FrzE-CheY.

tivity (Fig. 2, lane 3). However, when MBP:FrzE-CheY was mixed with FrzE or FrzE-CheA, the MBP:FrzE-CheY protein was phosphorylated (Fig. 2, lanes 4 and 5). The maltose binding protein domain which is present in MBP:FrzE-CheY does not appear to interfere with the availability of the CheY domain of FrzE to be phosphorylated by FrzE or FrzE-CheA. The results presented above show that the phosphorylation activity of the multidomain protein FrzE can be dissected into its two major components: the CheA-like autophosphorylating kinase, and a CheY-like domain which can accept phosphate group from either FrzE or FrzE-CheA.

The following control experiments support our phosphorylation experiments: We found that FrzE and FrzE-CheA were not phosphorylated when denatured by boiling, when Mn2+ was removed and when [α-^{32}P]ATP was used for in vitro labeling (data not shown). However, since we did not work with native proteins, it would be of interest to follow up this work with in vivo experiments.

FrzE is one of the first signal transduction proteins identified which incorporates the essential elements of both the sensor kinase and the response regulator in a single polypeptide. Recently many other proteins with similar structures were also identified in other signal transduction systems [4]. The results presented in this paper show that the homologies between FrzE and CheA and CheY represent not only similarities in protein sequence but also similarities in biochemical function. Similar results have also been found with other multidomain proteins [9,10]. These should be helpful in the understanding of the functioning of these proteins.

Acknowledgments: We thank Drs. Eldic Berger, Mark McBride, William McCleary, Thilo Köhler, Katheleen O'Connor, Karen Smith for helpful discussions. This work is supported by Public Health Science Grant GM 20509 from the National Institutes of Health.

References

[1] Shi, W., Köhler, T. and Zusman, D.R. (1993) Mol. Microbiol. 9, 601–611.
[2] Shi, W. and Zusman, D.R. (1994) Res. Microbiol. 145, 431–435.
[3] Parkinson, J.S. (1993) Cell 73, 857–71.
[4] Parkinson, J.S. and Kofoid, E.C. (1992) Annu. Rev. Genet. 26, 71–112.
[5] Matsumura, P., Roman, S., Volz, K. and McNally, D. (1990) in: Signaling Complexes in Bacterial Chemotaxis; Biology of the Chemotactic Response (Armitage, J.P. and Lackie, J.M., Ed.) pp. 135–154, Cambridge University Press, Cambridge.
[6] McCleary, W.R. and Zusman, D.R. (1990) Proc. Natl. Acad. Sci. USA 87, 5898–5902.
[7] McCleary, W.R. and Zusman, D.R. (1990) J. Bacteriol. 172, 6661–6668.
[8] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: Molecular Cloning: a Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[9] Yang, Y. and Inouye, M. (1991) Proc. Natl. Acad. Sci. USA 88, 11057–11061.
[10] Priestle, J.P., Grütter, M.G., White, J.L., Vincent, M.G., Kania, M., Wilson, E., Jardetzky, T.S., Kirshner, K. and Jansonius, J.N. (1987) Proc. Natl. Acad. Sci. USA 84, 5690–5694.